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14. ABSTRACT

TGF-β/Smad signaling plays crucial roles in breast cancer cell invasion and breast tumor metastasis. Understanding how TGF-β signaling is regulated in metastatic breast cancer cells will allow for development of novel targeted therapy and prognostic markers. In this research, we study the mechanisms through which 2 signaling molecules, G protein-coupled receptor kinase 2 (GRK2) and Breast Cancer Anti-estrogen Resistance 3 (BCAR3), antagonize Smad signaling in breast cancer cells. We also study for correlations between expressions of these factors in breast tumors to disease outcomes. Our results advance current understanding of an important aspect of breast cancer pathology, namely TGF-β-induced metastasis. They also define GRK2 and BCAR3 as novel prognostic markers of breast cancer disease relapse and metastasis.

15. SUBJECT TERMS
TGF-β, GRK2, BCAR3

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1. INTRODUCTION:

Transforming Growth Factor-beta (TGF-β) superfamily growth factors play crucial roles in mammary homeostasis by mediating cytostatic responses at different stages of mammary gland development [1]. TGF-β induces complex formation between its receptors, which are also serine and threonine kinases. Activated receptors phosphorylate and activate multiple effectors, including the Smad2 and Smad3 transcription factors which mediate a canonical TGF-β-Smad signalling axis [2]. In the mammary epithelium, Smad2 and Smad3 transactivate a panel of pro-apoptotic and cell cycle repressor genes to mediate TGF-β's cytostatic effects [3]. However, during breast cancer progression, genetic and epigenetic changes allow breast cancer cells to escape these effects. Eventually, the TGF-β-Smad signalling axis acts as a potent pro-metastatic mechanism [4]. In animal breast cancer metastasis models, interfering with key components of the TGF-β-Smad signalling axis slows down metastatic process [4, 5]. As such, understanding how TGF-β signalling is regulated in metastatic breast cancer cells will allow for development of novel targeted therapy and prognostic markers.

Smads, as transcription factors that translocate to the nucleus and bind to DNA, are difficult to be directly targeted by therapeutic agents. Therefore, we aim to study how Smad signaling can be modulated by intracellular signaling proteins. This study aims to elucidate the roles of 2 signaling molecules, G protein-coupled receptor kinase 2 (GRK2) and Breast Cancer Anti-estrogen Resistance 3 (BCAR3), as novel antagonistic factors of smad signaling. This work is a continuation of our previous studies showing that kinase GRK2 acts in a negative feed-back loop manner to inhibit the TGF-β-Smad signalling axis [6].

2. BODY

During the first funding year, we established stable cells expressing Smad phosphor-mimic mutants and showed that the mutants could antagonize the TGF-β-Smad signaling axis (data included in previous report). In addition, we found that GRK2 expression levels, combined with that of BCAR3, could predict favourable metastasis-related disease outcomes (data included in previous report). The following report summarizes our further findings based on the work in the first year.

2.1. Generating stable cell lines overexpressing BCAR3 and GRK2.

The following studies address Task 1 in the SOW. We are in a process to generate and validate stable cell lines overexpressing BCAR3, GKR2 and GRK2 K220M (kinase dead form). We have established pools of SCP2 cells that are resistant to antibiotic selection. The expression levels of ectopic proteins are however low. At the moment we are in a process to repeat stable transfection and will aim to screen for stable colonies. Besides using SCP2 cells, as originally proposed, we also included a newly characterized invasive breast cancer cell line, SUM-159-PT. These cells express low level of BCAR3 among a list of breast cancer cell lines that we tested (Figure 1), and carries active response to TGF-β as determined by Smad activation, cell migration and invasion (data not shown).

2.2. BCAR3 regulates TGF-β induced breast cancer cell migration and invasion.

Section 2.2 and Section 2.3 address Task 2 in the SOW. We have validated siRNA constructs that can efficiently knock down GRK2 and BCAR3 (data not shown). Transfecting BCAR3 siRNA into SCP2 cells resulted in a significant decrease of TGF-β-induced invasion through Matrigel (Figure 2). On the other hand, in order to perform transwell invasion assay with a gain-of-function approach, we will need stable clones of cells. Transient expression does not serve for this purpose since not all cells will be transfected. As stated in Section 2.1, we are still in a process to generate stable clones of cells over expressing GRK2 and BCAR3.

2.3. BCAR3 antagonizes Smad signaling transduction.

Based on our previous findings that suggest a cooperative function between GRK2 and BCAR3, we tested whether BCAR3 can antagonize the canonical TGF-β/Smad signaling axis. Our data suggest that ectopic BCAR3 expression in 2 breast cancer cell lines that express relatively low amount of BCAR3 (SUM-159-PT and MCF-7) antagonizes Smad activation, nuclear translocation and Smad-mediated gene transcription (Figure 3A-D and Figure 4A). Consistently, knocking down endogenous BCAR3 in SCP2 cells, which express a high level of BCAR3, potentiates Smad signaling (Figure 4B).

To further develop the proposed studies in Task 2, we started to elucidate a detailed mechanism through which BCAR3 antagonizes Smad signaling. Smad signaling transduction requires interaction between TGF-β receptors, as well as interactions between Smad and other signaling molecules such as Smad Anchor of Receptor Activation (SARA) and Smad4. Our preliminary data suggest that BCAR3 promote an interaction between Smad and p130Cas, a signaling docking molecule previously shown to directly bind to Smad2 and Smad3 (Figure 5). We are currently studying whether BCAR3 requires p130Cas to antagonize Smad signaling, and whether BCAR3 expression interferes the interactions between Smad and key signaling molecules such as TGF-β type I receptor, SARA and Smad4.

2.4. Accomplishments towards training

The reviewer suggested it is important to include information regarding training accomplishments, which was missing in the first report. This section summarizes training accomplishments in the first 2 funding years.

I have completed all required Ph.D. coursework and passed the examination to receive Ph.D. candidacy. Therefore, the traineeship supported me to obtain additional trainings that are not available at McGill University. In the first funding period, I used the funding for workshop to attend an intensive course on Functional Genomics and System Biology at the Sanger Institute. I was selected to be one of the 18 trainees from applicants worldwide. While attending the course, I performed a series of cutting-edge techniques, such as RNA-seq, microRNA-seq, RNAi library screening and Illumina microarray, under direct supervision of leading scientists and developers of these techniques. I learnt about the power and limitations of these novel methods, and also acquired basic programming skills in analysing and visualizing data generated by these methods. Upon completing the course, I applied this learning experience directly in my current research, as I was able to analyse existing microarray data in our lab and performed data mining in functional genomics databases to identify BCAR3 as a novel TGF-β target that may be functionally coupled to GRK2. In addition, this experience also helps me to define an

interest in computational biology and a wish to integrate computational skills to bench-work in further post-doctoral training.

In the past 2 years, travel allowance of the traineeship, together with additional support from McGill University, allowed me to give poster presentations in three international conferences with highly-defined topics relevant to cancer biology, namely the Cold Spring Harbor Laboratory Conference on Metastasis and Cancer Modelling (April 2011), AACR Conference on Tumor Microenvironment (November 2011, Orlando) and AACR Conference on Drug Resistance in Cancer (June 2012). These were great opportunities to present my work and receive comments. In addition, I also had valuable networking opportunities. I discussed with attendants of the conferences who have similar interests, and have set up collaborations to share reagents and expertise. I also discussed with 2 world-leading breast cancer researchers, Dr. Joan Massague and Dr. Joan Brugge regarding potential post-doctoral training opportunities.

3. KEY RESEARCH ACCOMPLISHMENTS:

1. Establish proteasome degradation as a mechanism by which GRK2 antagonizes Smad signalling.
2. Identify BCAR3 as an endogenous antagonist of TGF-β signaling and biological response in breast cancer cells.
3. Generate and validate breast cancer cell lines overexpressing BCAR3 and GRK2.

4. REPORTABLE OUTCOMES:

Poster Abstracts:

3. Guo J and Lebrun JJ, Breast Cancer Anti-Estrogen Resistance 3 Antagonizes Transforming Growth Factor β signaling and is Correlated to Favorable Disease Outcomes in Breast Cancer Patients. AACR Conference on Mechanism of Resistance to Molecular Targeted Therapies, San Diego CA, USA, May 9-12 2012.
2. GRK2 antagonizes pro-malignant properties of breast cancer cells and pro-angiogenic properties of vascular smooth muscle cells. AACR Conference on Tumour Microenvironment Complexity: Emerging Roles in Cancer Therapy. November 3-6, Orlando FL
1. BCAR3 regulates TGF-β-induced cell signalling in breast cancer cells. Cold Spring Harbour Laboratories Conference on the Biology of Cancer: Microenvironment, Metastasis & Therapeutics. April 26-30, Cold Spring Harbour NY

5. CONCLUSION:

5.1 Discussion of results

This year we attempted to generate breast cancer cells that stably express GRK2 and BCAR3. With these cells we will be able to better study the biological functions of these factor, particularly, how they can affect cellular response to TGF-β. We started with generating a pool of cells. The advantage of this approach is to allow for distinguishing functions of GRK2 and BCAR3 from colony-specific response. As the expression levels of ectopic proteins in the pools of the cells were not optimal, we have changed out plan and are currently screening for clones that express high levels of GRK2 and BCAR3.

At the same time, we performed a thorough study on how BCAR3 could modulate TGF-β/Smad signaling. By investigating into each key steps of Smad signaling transduction, we found that BCAR3 interferes with Smad activation. These findings provide a mechanism of a novel function of BCAR3. We also obtained preliminary data suggest a role of p130Cas in this novel signaling event. Traditionally, BCAR3 is viewed to be associated with aggressive disease phenotypes as its ectopic expression in estrogen-dependent breast cancer cells allows the cells to proliferate under the presence of Tamoxifen. Our data challenge this view and suggest the research community to re-examine the function and prognostic value of BCAR3 in breast cancer.

5.2 Implications of the study

Together with previous work, our data suggest that both GRK2 and BCAR3 are endogenous factors in breast cancer cells that negatively regulate the canonical TGF-β/Smad signaling axis. As cytoplasmic signaling molecules whose activities are modulated by kinases and phosphatases, GRK2 and BCAR3 are potentially drugable. In addition, our findings in the first funding year also suggest prognostic values of these factors to predict breast cancer relapse and metastasis.

6. REFERENCES:

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2. Shi, Y. and J. Massague, *Mechanisms of TGF-beta signaling from cell membrane to the nucleus.* Cell, 2003. **113**(6): p. 685-700.
3. Dzwonek, J., et al., *Smad3 is a key nonredundant mediator of transforming growth factor beta signaling in Nme mouse mammary epithelial cells.* Mol Cancer Res, 2009. **7**(8): p. 1342-53.
4. Kang, Y., et al., *Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway.* Proc Natl Acad Sci U S A, 2005. **102**(39): p. 13909-14.
5. Tian, F., et al., *Smad-binding defective mutant of transforming growth factor beta type I receptor enhances tumorigenesis but suppresses metastasis of breast cancer cell lines.* Cancer Res, 2004. **64**(13): p. 4523-30.
6. Ho, J., et al., *The G protein-coupled receptor kinase-2 is a TGFbeta-inducible antagonist of TGFbeta signal transduction.* EMBO J, 2005. **24**(18): p. 3247-58.

7. SUPPORTING DATA:

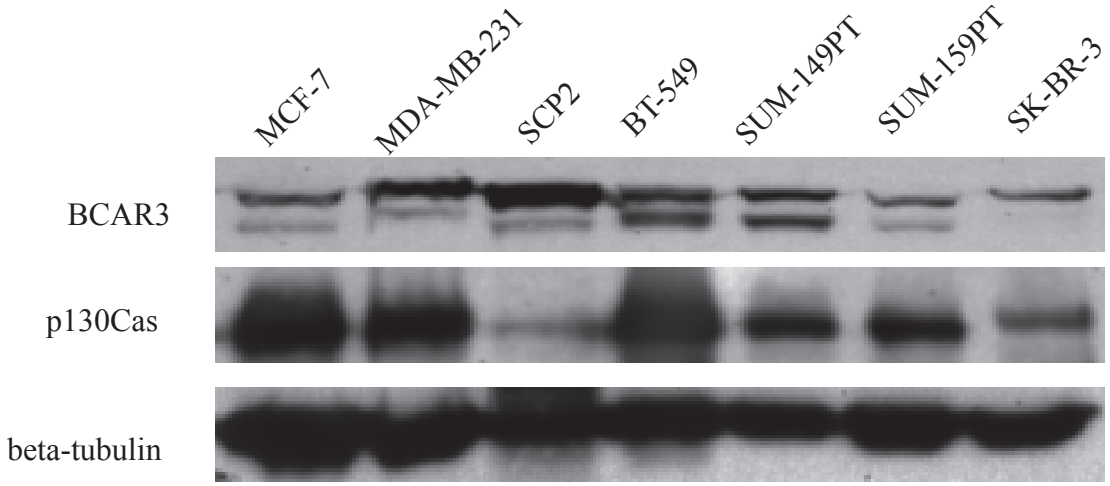

Figure 1. BCAR3 and p130Cas protein expression in human breast cancer cells.

Expression levels of p130Cas and BCAR3 were surveyed in breast cancer cells representing luminal (MCF-7), basal/mesenchymal-like (231, SCP2 and BT549) and Her2+ (149, 159, SK-BR-3) breast cancer cells. Sum-149 cells carry inactivated Her2.

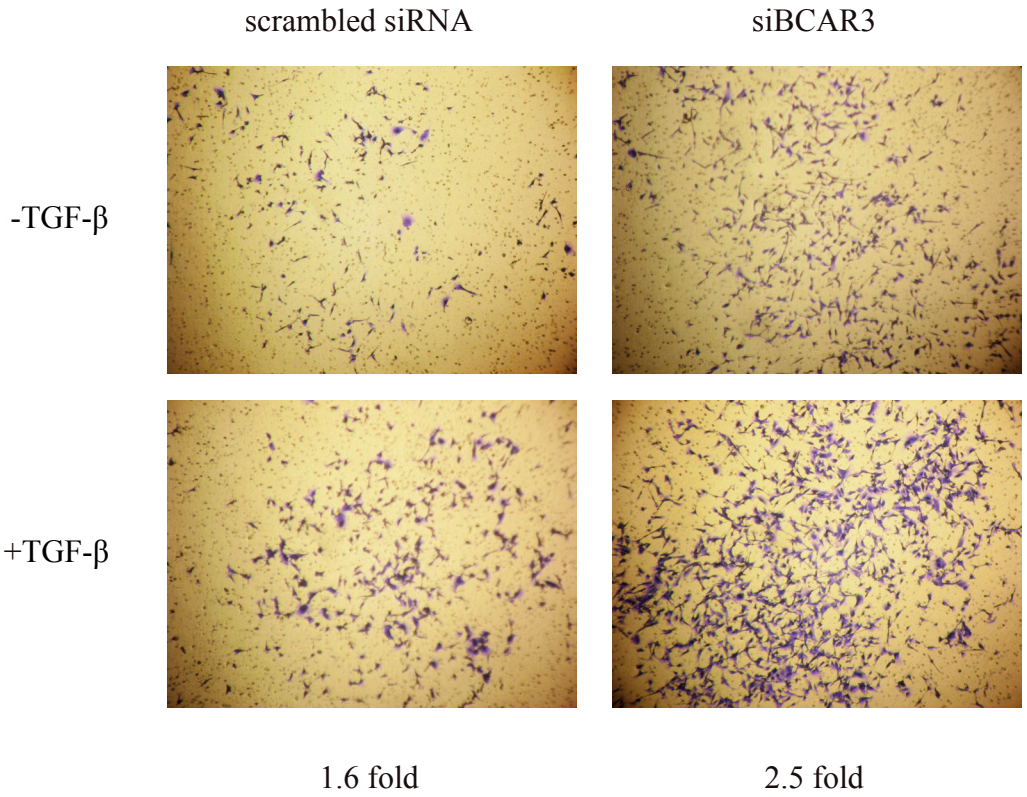

Figure 2. Knocking down endogenous BCAR3 potentiates TGF-β- inducded breast cancer cell invasion.

SCP2 cells were transfected with scrambled siRNA or siRNAs targeting BCAR3 for 72 hours. Cells were then stimulated with or without 100 pM TGF-β, and subjected for transwell invasion assays. Figures represent results of at least 3 independent experiments.

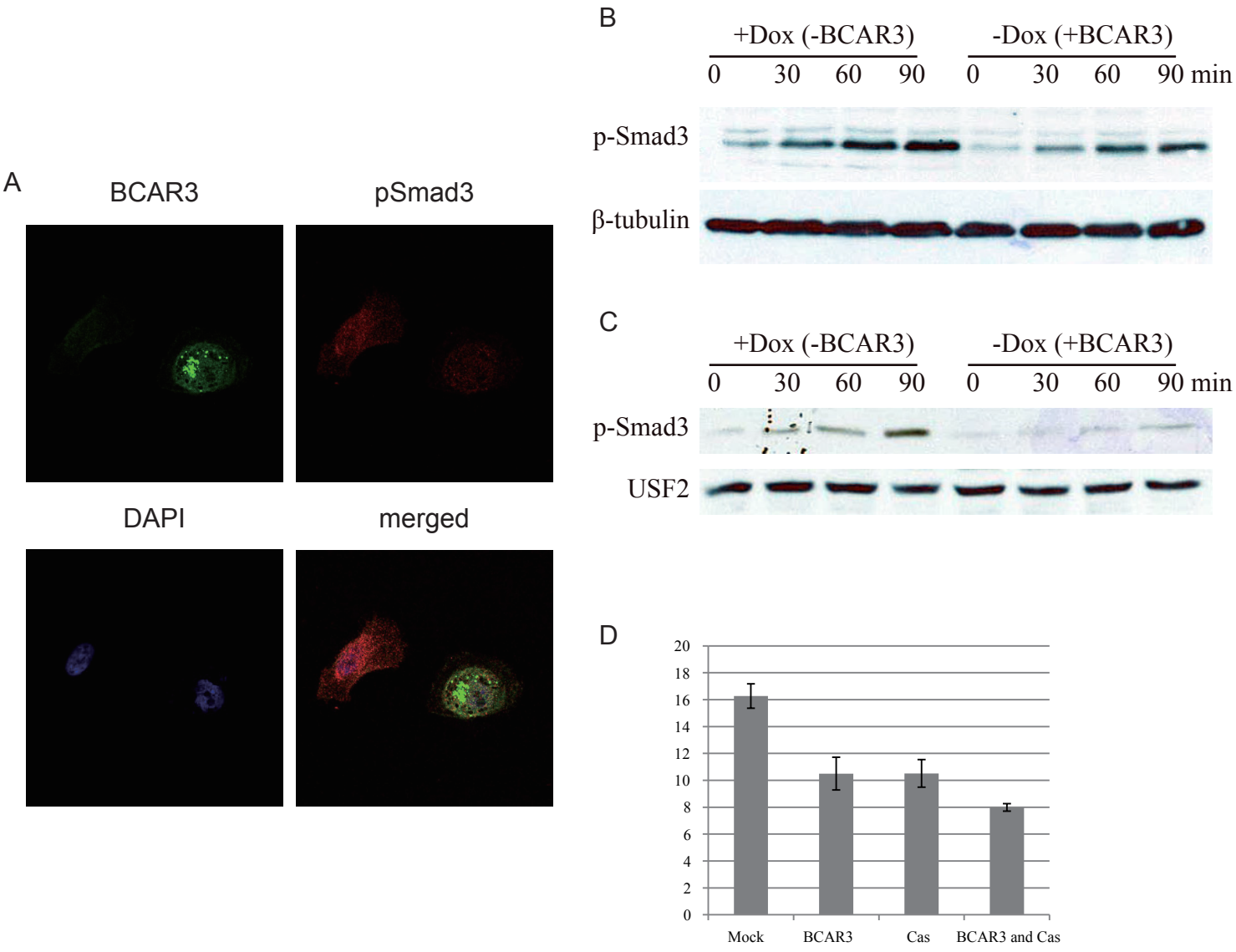

Figure 3. BCAR3 antagonizes Smad signaling.

A: SUM-159-PT breast cancer cells were transfected with FLAG-tagged rat AND-34/BCAR3. Cells were then starved overnight and stimulated with 100pM TGF-β. Presence of BCAR3 in transfected cells and activation of Smad3 was analysed using immunofluorescence staining using antibodies specific for FLAG and C-terminal serine phosphorylated Smad3, respectively. Images were taken using Zeiss LSM780 confocal microscopy under a 63X oil objective. The image is a representitive picture of more than 10 fields.

B: Tet-off BCAR3 inducible MCF-7 cells were cultured without or with doxycycline for 96 hours. Cells were starved in these conditions for 24 hours and stimulated with 100pM TGF-β. Activation of Smad3 was analysed using immunoblotting.

C: Tet-off BCAR3 inducible MCF-7 cells were cultured without or with doxycycline for 96 hours. Cells were starved in these conditions for 24 hours and stimulated with 100pM TGF-β. Nuclear extracts were prepared and phosphorylatd Smad3 in the nucleus was analysed using immunoblotting.

D: SUM-159-PT cells were transfected with FLAG-tagged rat AND-34/BCAR3, GST-tagged p130Cas, together with a Smad-responsive 12CAGA-lux luciferase reporter construct and a β-galactosidase reporter construct. Cells were then starved overnight and stimulated with 100pM TGF-β. Smad-dependent transcription was determined by luciferase assay and expressed as fold change. Error bars show standard deviation of the mean of3 independent experiments.

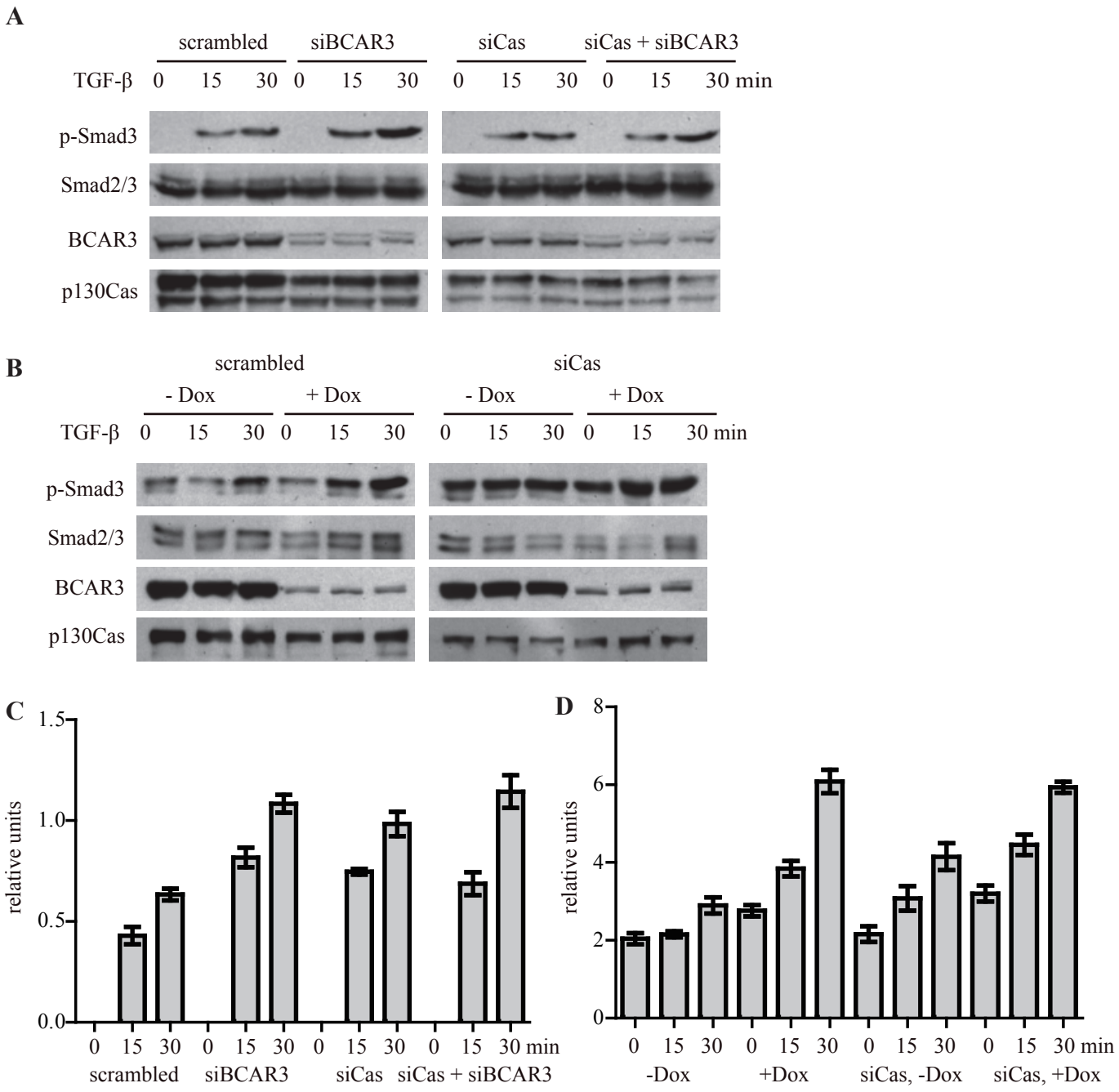

Figure 4. BCAR3 antagonizes Smad activation.

A: SCP2 breast cancer cells were transfected with 25nM BCAR3 siRNA or a scrambled control (left panel), or co-transfected with 25nM Cas siRNA and BCAR3 siRNA or control (right panel). Cells were then starved over-night and stimulated with 100pM TGF-β. Activation of Smad3 was analysed using immunoblotting with an antibody specific for C-terminal serine phosphorylated Smad3.

B: Tet-off BCAR3 inducible MCF-7 cells were cultured without or with doxycycline for 72 hours. Cells were starved in these conditions for 24 hours and stimulated with 100pM TGF-β. Activation of Smad3 was analysed using immunoblotting.

C,D: Quantification for phospho-Smad3 signal in A and B, respectively. Pixel density of each band on the phospho-Smad3 blots and the total Smad2/3 blots is quantified using QuantityOne software (Bio-Rad). The density of each phospho-Smad3 bands is normalized using the density of the corresponding Smad3 band (lower band of the doublet) and expressed as relative units. Figures show quantification of 3 experiments with standard error of the mean.

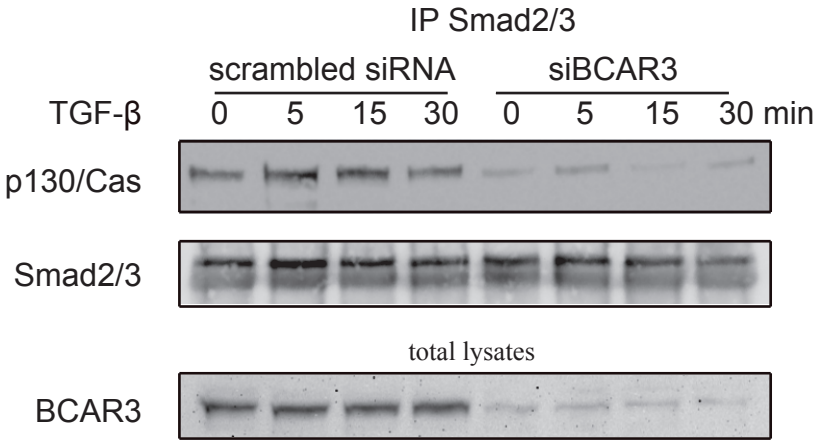

Figure 5. Endogenous BCAR3 promotes an interaction between Smad2/3 and p130Cas.

SCP2 cells were transfected with scrambled siRNA or siRNA targeting BCAR3. Smad2 and Smad3 in total cell lysates were immunoprecipitated using a rabbit polyclonal antibody. Amount of p130Cas in the precipitant was determined by Western blotting.